

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Shi-You Ding, et al. Docket No.: NREL 01-36  
Serial No.: 09/917,376 Art Group: 1633  
Filed: July 28, 2001  
Title: THERMAL TOLERANT AVICELASE FROM ACIDOTHERMUS  
CELLULOLYTICUS



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**AMENDMENTS -- MARKUP**

**IN THE SPECIFICATION:**

Please replace the third full paragraph on page 3, beginning line 27, with the following:

There is a need within the art to generate alternative cellulase enzymes capable of assisting in the commercial-scale processing of cellulose to sugar for use in biofuel production. Against this backdrop the present invention has been developed. The potential exists for the successful, commercial-scale expression of heterologous cellulase polypeptides, and in particular novel cellulase polypeptides with or without any one or more desirable properties such as thermal tolerance, and partial or complete resistance to extreme pH[acid] inactivation, proteolytic inactivation, [and ]solvent inactivation, chaotropic agent inactivation, oxidizing agent inactivation, and detergent inactivation. Such expression can occur in [filamentous ]fungi, bacteria, and other hosts.

Please replace the second full paragraph on page 4, beginning line 11, with the following:

The present invention provides AviIII, a novel member of the glycoside hydrolase (GH) family of enzymes, and in particular a thermal tolerant glycoside hydrolase useful in the degradation of cellulose. AviIII polypeptides of the invention include those having an amino acid sequence shown in SEQ ID NO:1, as well as polypeptides having substantial amino acid sequence identity to the amino acid sequence of SEQ ID NO:1 and useful fragments thereof, including, a catalytic domain having significant sequence similarity to the GH74 family, a [first carbohydrate binding domain (type II) and a second] carbohydrate binding domain (type III). See FIG 1.

Please replace the third full paragraph on page 13, beginning line 19, with the following:

"Thermal tolerant" refers to the property of withstanding partial or complete

inactivation by heat and can also be described as thermal resistance or thermal stability. Although some variation exists in the literature, the following definitions can be considered typical for the optimum temperature range of stability and activity for enzymes: psychrophilic (below freezing to 10°C); mesophilic (10°C to 50°C); thermophilic (50°C to 75°C); and caldophilic (75°C to above boiling water temperature). The stability and catalytic activity of enzymes are linked characteristics, and the ways of measuring these properties vary considerably. For industrial enzymes, stability and activity are best measured under use conditions, often in the presence of substrate. Therefore, cellulases that must act on process streams of cellulose must be able to withstand exposure up to thermophilic or even caldophilic temperatures for digestion times in excess of several hours.

Please replace the third full paragraph on page 15, beginning line 23, with the following:

[Cellulases belong to the GH family of enzymes.] Cellulases are produced by a variety of bacteria and fungi to degrade the beta-(1,4)-[ $\beta$ -1,4]glycosidic bond of cellulose and to so produce successively smaller fragments of cellulose and ultimately produce glucose. At present, cellulases are found within are at least 11 different GH families. Three different types of cellulase enzyme activities have been identified within these GH families: exo-acting cellulases which cleave successive disaccharide units from the non-reducing ends of a cellulose chain; endo-acting cellulases which randomly cleave successive disaccharide units within the cellulose chain; and  $\beta$ -glucosidases which cleave successive disaccharide units to glucose (J. W. Deacon, (1997) Modern Mycology, 3rd Ed., ISBN: 0-632-03077-1, 97-98).

Please replace the fifth full paragraph on page 16, beginning line 31, with the following:

As described more fully in the Examples below, [AviIII, a novel thermostable cellulase,] has now been identified and characterized. The predicted amino acid sequence of AviIII (SEQ ID NO:1) has an organization characteristic of a cellulase enzyme. AviIII contains a [carbohydrate binding domain - linker domain - ]catalytic domain -[linker domain- fibronectin domain - linker domain -] carbohydrate binding domain unit. In particular, AviIII includes a [carbohydrate binding domain type III (CBDIII) (amino acids from about A35 to about A187), a] GH74 catalytic domain (amino acids from about A37 to about G776 [N231 to about P870), and a CBD<sub>II</sub> (amino acids from about G1021

to about S1121)] and a carbohydrate binding domain type III (CBDIII) (amino acids from about V859 to about at least Q946).

Please replace the second full paragraph on page 17, beginning line 15, with the following:

AviIII, as noted above, has a catalytic domain, identified as belonging to the GH74 family. [The GH74 domain family includes an avicelase from *Aspergillus aculeatus* number of exoglucanases, for example, from *Cellulomonas fimi*, and exoglucanase E3 isolated from *Thermobifida fusca*. The GH74 members degrade substrate using an inverting mechanism. (Being a member of the GH74 family of proteins identifies AviIII as potentially having cellulase activity)].

Please replace the second full paragraph on page 18, beginning line 34, with the following:

As listed and described in Tables 1 and 5, the isolated AviIII polypeptide includes an N-terminal hydrophobic region that functions as a signal peptide, having an amino acid sequence that begins with Met1 and extends to about A36[4; a carbohydrate binding domain having sequence similarity to such type III domains that begins with about A35 and extends to about A187], a catalytic domain having significant sequence similarity to a GH74 family domain that begins with about A37[N231] and extends to about G776, [P870, a fibronectin type III domain that begins with about D901 and extends to about G985,]a carbohydrate binding domain having sequence similarity to such type III domains that begins with about V859 and extends to about at least Q946 [type II region that begins with about G1021 and extends to about S1121]. Variants and derivatives of AviIII include, for example, AviIII polypeptides modified by covalent or aggregative conjugation with other chemical moieties, such as glycosyl groups, polyethylene glycol (PEG) groups, lipids, phosphate, acetyl groups, and the like.

Please replace the third full paragraph on page 32, beginning line 24, with the following:

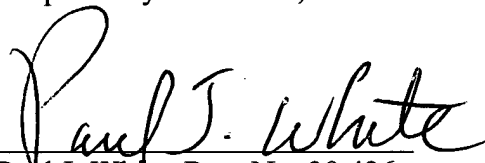
Sequencing data from primer walking and subclones were assembled together to verify that all SL-3 regions had been sequenced from both strands. An open reading frame (ORF) was found in the 9 kilobase Bam H1 fragment, C-terminal of E1 (U.S. Patent 5,536,655), termed AviIII. An ORF of about 3 kb [3366 bp] [SEQ ID NO:2] and deduced amino acid sequence [SEQ ID NO:1] are shown in Tables 1 and 2. The amino

acid sequence predicted by SEQ ID NO:1 was determined to have significant homology to known cellulases, as is shown below in Example 2 and Table 3.

IN THE CLAIMS:

4. The composition of claim 1, 2, or 3 wherein the carbohydrate binding domain (CBD) III of the thermostable AviIII peptide is further defined as comprising a length of about 80 to about 160[150] amino acids.
5. The composition of claim 1, 2, 3, or 4 wherein the carbohydrate binding domain (CBD) III of the thermostable AviIII peptide is further defined as comprising a length of about 154[90] amino acids.

Respectfully Submitted,

A handwritten signature in cursive script, reading "Paul J. White". The signature is written in dark ink and is positioned above a horizontal line.

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Dated: October 30, 2001.

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